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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 27/333 — A1

(11) International Publication Number: WO 94/25862

(43) International Publication Date: 10 November 1994 (10.11.94)

(21) International Application Number: PCT/US94/04883

(22) International Filing Date: 3 May 1994 (03.05.94)

(30) Priority Data:
08/057,275 4 May 1993 (04.05.93) US

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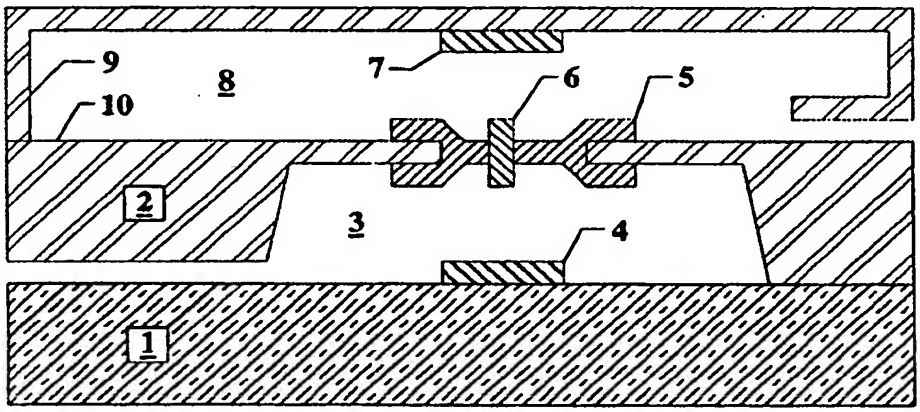
(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: BIOSENSOR SUBSTRATE FOR MOUNTING BILAYER LIPID MEMBRANE CONTAINING A RECEPTOR



(57) Abstract

A biosensor substrate is described which comprises: (a) a body (2) having side walls (9) and a solid floor (10) defining a chamber (8) to hold a first solution; (b) at least one open aperture in the solid floor to allow for the structural integrity of a bilayer lipid membrane (5) containing an analyte receptor (e.g., an ion channel receptor) (6) across the aperture; and (c) a chamber (3) below the solid floor for a second solution in contact with the membrane containing the receptor.

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BIOSENSOR SUBSTRATE FOR MOUNTING BILAYER LIPID MEMBRANE CONTAINING A RECEPTOR

BACKGROUND OF THE INVENTION

The present invention relates to a substrate for a biosensor which incorporates a receptor into a suspended lipid bilayer membrane. The term "biosensor" as used herein is intended to broadly cover devices that use a biologically sensitive material or "receptor" which reacts to stimulus and which can be used, for example, in the detection of chemicals or analytes. Since the cell membranes of both plants and animals are composed of lipids in the form of a lipid bilayer and since various proteins that perform vital cell functions span such a bilayer structure, much work has been performed on model membrane structures. In general, bilayer lipid membranes (BLM), that are not in the form of a liposome, can be divided into suspended BLM's, where the lipid bilayer is positioned between two aqueous solutions, and supported BLM's where the lipid bilayer is placed on a solid support. The present invention relates to the first type of configuration. The suspended BLMs known heretofore were stable up to only about two hours as contrasted with supported BLMs which are stable for up to about seventy-two hours in one study (but which yield differing specific capacitance values from suspended BLMs due to difficulties in measuring the specific capacitance and resistance). Liposomes require lengthy techniques for preparation and are less uniform and flexible as compared to suspended BLMs. Supported BLMs offer little or no control over the electrochemical properties of one side of the membrane thus not providing a feasible environment for the incorporation of a variety of biological receptors as compared to the suspended BLMs.

The incorporation of receptors in the BLM for the detection of an analyte is also a known technique. The following illustrate certain work of this type:

1. M. Thompson et al. in Analytical Chemistry, 1982, 54, 76-81 discuss the use of various microfiltration filters as supports for bilayer lipid membranes.

2. U. J. Krull and coworkers in U.S. Patent No. 4,661,235 illustrate supporting a bilayer lipid membrane using a circular aperture in a sheet of TEFLON fluoropolymer dividing two separate chambers in a housing.

3. U.S. Patent No. 4,874,499 to R. L. Smith et al. illustrates electrochemical microsensors and illustrates (in Fig. 3b) a device having an ion selective "polymeric" membrane, which acts as an ion sensor, placed in a funnel-shaped opening above a cavity holding a silver-silver chloride electrode over a field effect transistor (FET). The structure shown in this patent possesses a funnel shaped geometry having a rectangularly shaped aperture. Apertures that are used in bilayer membrane work are circular in shape for proper support of the lipid bilayer.

4. U.S. Statutory Invention Registration No. H201 to P. Yager describes biosensors comprising membrane proteins (e.g., the acetylcholine receptor protein) reconstituted in polymerized lipid bilayers which are supported in a patch clamp-arrangement adjacent internal and external aqueous compartments that are separated by a glass microelectrode tip.

5. U.S. Patent No. 5,001,048 to R. F. Taylor et al. illustrates an electrical biosensor containing a biological receptor immobilized and stabilized in a protein film. The protein film is sandwiched between a silica substrate, on one side, and a laminate comprising an equipotential barrier, a reference membrane, and a second silica support, on the other side.

6. U.S. Patent No. 5,111,221 to T. L. Fare et al. also illustrates a receptor-based sensor in which a lipid bilayer containing receptor(s) is deposited onto a porous silicon substrate.

SUMMARY OF THE INVENTION

The present invention relates to a biosensor substrate, adapted to mount a bilayer lipid membrane which contains at least one receptor for use in the detection of an analyte in solution, which comprises:

(a) a body having side walls and a solid floor which define a chamber to hold a first solution;

(b) at least one open aperture in the solid floor which is of suitable dimensions to allow for the structural integrity of the bilayer lipid membrane comprising the receptor when the membrane is placed across the open aperture in contact with the first solution; and

(c) a chamber below the solid floor adapted to hold a second solution in contact with the membrane.

DESCRIPTION OF THE DRAWINGS

The present invention is further understood by reference to the Drawings, which form a part of the present specification wherein,

Fig. 1 is a cross-sectional view of a biosensor which can be used for monitoring the concentration of chemicals in a solution;

Fig. 2 is a view similar to that in Fig. 1 which uses a polyimide diaphragm for support of the membrane.

Figs. 3 and 4 are views showing the aperture in the polyimide diaphragm or septum over the silicon substrate;

Figs. 5A - 5E illustrate the microfabrication process for making the polyimide diaphragm or septum;

Fig. 6 illustrates the electrical measurement set-up described in the Example;

Figs. 7A -7D illustrate alamethicin activity recorded at +150 mV holding potential; and

Figs. 8A - 8D show acetylcholine channel receptor (AChR) activity recorded under +150 mV holding potential.

DESCRIPTION OF PREFERRED EMBODIMENTS

A biosensor containing the biosensor substrate disclosed herein can be made specifically sensitive to a wide variety of biological or chemical materials. The biosensor comprises a detector portion and a transducer. The detector recognizes the desired analyte(s) in the adjacent environment using a suitable receptor, embedded in a bilayer lipid membrane, which generates a signal upon detection of the target species. A preferred class of receptor are several ion channel forming proteins. The function of the transducer portion of the biosensor is to couple the detected signal into a suitable storage unit, such as a personal computer. The transducer component of the biosensor consists of the physical unit (or "substrate") carrying the bilayer lipid membrane, the compartment(s) for the bathing solution, and the electrodes to monitor current generated.

Preferably, a biosensor should be highly specific and sensitive towards the target analyte(s), should be durable, miniaturizable and easily interfaced with the selected measurement and data storage units. In a BLM system, the specificity is dependent upon the receptor chosen for incorporation in the BLM and the inertness of the lipid material to the target analyte(s). The sensitivity, on the other hand, is dependent upon the receptor protein used as

well as the measurement system that is selected. Although either amperometric, potentiometric, or optical measurement modes can be selected, it is preferred to select the amperometric mode which has the highest inherent sensitivity (10^{-7} to 10^{-8} mole/liter).

Figs. 1 and 2 illustrate preferred embodiments of the present biosensor substrate.

The biosensor substrate comprises a body 2 which has side walls 9 and a floor 10 which define a chamber 8 adapted to hold an aqueous solution. In a preferred embodiment the body 2 is formed of micromachined silicon with the side walls being formed of glass. A glass shield structure 1 is placed beneath body 2 to enclose a second chamber 3 which is adapted to also hold an aqueous solution. These glass structures 1 and 9 provide access to suitable electrodes 4 and 7 which can be silver-silver chloride electrodes. The bilayer lipid membrane 5 contains reincorporated active membrane proteins, for example, an ion channel forming protein such as a nicotinic acetylcholine receptor 6 and spans an aperture in the floor 10. It preferably is round (or circular) with a diameter of from about 1 μm to about 50 μm.

Fig. 2 shows an analogous structure in which chambers 13 and 18 for the aqueous solutions are separated by a micromachined silicon body 12, for example, having, as the floor 20, a polyimide which contains the bilayer lipid membrane 15 and incorporated receptor 16. Glass side wall shield 19 and glass shield 11 enclose the respective aqueous chambers or compartments 18 and 13 and provide access to the electrodes 17 and 14. The use of polyimide as a support for the bilipid membrane has several advantages. First, the sealing obtained in a device that uses polyimide as the membrane support is appreciably better as compared to a device using a silicon membrane support as depicted in Fig. 1. Second, since polyimide is hydrophobic, silanization,

which is required for silicon devices, is not needed thereby eliminating the time consuming and tedious silanization process required for silicon membrane support structures. Third, polyimide is transparent to light which lends to use of optical detection of molecular binding events, for example, by coupling light from a fiber into the polyimide film to excite the bilipid membrane with measurement of the optical response of the membrane to detect analyte-receptor interactions.

The biosensor substrate of this invention allows for the fabrication of a compact, solid state biosensor which can detect the presence of environmental stimulus by having electrodes detect voltage and current changes across a reconstituted bilayer lipid membrane containing receptor proteins therein which react to a specific stimulus. The support structure extends the longevity of the lipid bilayer component used in such a biosensor. Since the lipid membrane is formed on the same substrate along with portions of the electronic circuit that monitors and records the electrical activity from the membrane-receptor combination, there is good compatibility between the electronic and biological detector subcomponents.

The present invention is illustrated by the Examples which follow.

EXAMPLE

Materials and Methods

Materials

Photosensitive polyimide (Pyralin PI2722 brand) was obtained from DuPont Electronics (Wilmington, DE). The developing solutions were mixtures of γ-hydroxybutyric acid (obtained from Sigma, St. Louis, MO), and xylenes (from Baker Analyzed, Phillipsburg, NJ). Black Teflon fluoropolymer for two detachable chambers (for the trans and cis sides of the BLM) was purchased from Laird Plastic (Spokane, WA). Silicon substrate carrying the polyimide septum was attached to one of the detachable chambers with Teflon epoxy (Duralco 4540 brand obtained from Cotronics Co., Brooklyn, NY). Parts of the electrical measurement set-up, namely a CV-201 brand current to voltage (I/V) converter headstage, Axopatch 200 brand patch-clamp amplifier and TL-1 A/D brand converter were obtained from Axon Instruments (Foster City, CA). Lecithin type II-S, n-decane, and alamethicin were purchased from Sigma (St. Louis, MO).

Fabrication of polyimide apertures

A schematical diagram of the designed polyimide septum is shown in Fig. 3. The silica layer between the silicon and polyimide is a sacrificial layer that is used to protect the polyimide from the anisotropic KOH etchant. The aim was to obtain an aperture with diameter, R, equal to 50 μm, and an aspect ratio of about 0.1. The microlithography steps used in the fabrication process of polyimide septa are outlined in Fig. 4. A contact printing technique was used throughout the process and is described in detail by W. R. Runyan et al., Semiconductor Integrated Circuit Processing Technology (Addison Wesley, Reading PA, 1990). First, n-type <100> oriented 3 inch silicon (Si) wafers were agitated in a

sulfuric acid•hydrogen peroxide solution for ten minutes. After a through rinse under deionized water (DIW), they were cleaned of organic contaminants by rinsing under trichloroethylene, acetone, methanol, and finally under DIW. Next, 1 μm thick silica layers were thermally grown on both sides of the wafers. Polyimide stock solution was thawed to room temperature before each process run. Polyimide was spun on the front surface of a silicon wafer for forty seconds, at 4000 rpm, forming a uniform layer. After a pre-curing period of three minutes at 85°C, the polyimide layer was exposed for one minute using a 450 nm UV light source. Four developer solutions with volume ratios 5:5, 6:4, 7:3, 8:2 of xylenes:γ-hydroxybutyric acid were prepared and sprayed on the exposed surface with durations of twenty, sixteen, twelve and eight seconds, respectively. During the development, unexposed (masked) regions were removed by the solution. Final curing was done in a furnace specifically designed for polyimide, first at 150°C for one-half hour, and then at 350°C for one hour (under N_2 gas). The final curing is a critical step since it determines the characteristics of the aperture. Next, a window pattern was defined over the back surface silica for anisotropic etching. Since the anisotropic solution dissolves polyimide, it was necessary to protect it with an asphaltum coating. The anisotropic etching solution consisted of 50 gm potassium hydroxide, 100 ml water, and 50 ml methanol. The etching rate was observed to be 1 μm per minute at 85°C. Finally, the sacrificial silica was etched using buffered hydrofluoric acid. The process described herein is compatible with standard microelectronic fabrication.

Electrical measurement set-up and detachable Teflon chambers

The electrical measurement set-up is shown in Fig. 5 and is very similar to the design given by Alvarez in Ion Channel

Reconstitution (Plenum Press, New York, 1986) pp. 115-130. The fluoropolymer cuvette was basically a detachable unit of two pieces. The silicon partition carrying the polyimide septum was fixed on one piece using Duralco 4540 brand Teflon fluoropolymer epoxy. An optional silicon sealant layer was applied to the surface to reduce the force on silicon chips during attachment. After proper curing of both adhesives, the two Teflon fluoropolymer units were snugly fit together using long screws with the polyimide septum in between. The CV-201 brand apparatus is a capacitive headstage for current-voltage (I/V) conversion and the Axopatch 200 apparatus is a patch-clamp amplifier which differentiates the headstage output signal, filters it and finally amplifies the signal. The waveform generator gives a 125 Hz, 20 mV_{p-p} triangular waveform that was used in the specific capacitance and resistance measurements. The TL-1 A/D brand converter provided digitized forms of Axopatch 200 measurements to a IBM PC-compatible computer.

Lipid solutions, Alamethicin channels, and AChR vesicles

The standard lipid solution was 10 mg of lecithin type II-S in 1 ml of n-decane, and was prepared fresh every day. This solution was agitated on a vibrating plate until turbidity was gone. Alamethicin was dissolved in 100% ethanol (500 ng/ml) and stored at 2°C. This solution was also agitated before each use. Vesicles (or liposomes) carrying the acetylcholine ion channel receptors (AChRs) were prepared by a technique similar to the one described by Lindstrom et al. (1980). They were stored at -20°C and sonicated for about five minutes before each use.

Results

Polyimide septa on silicon substrates

The polyimide septum supported on the silicon rim contained an aperture with a diameter of about 40 μm and a thickness of about 6 μm. Thus, an aspect ratio of about 0.1 was obtained which is suitable for stirring purposes. The extremely smooth tapered aperture edges and septum surface were evident in micrographs taken of the structures, were formed as a result of the UV microlithography procedure and are important features for long term stability of the lipid bilayer. The polyimide septum was mechanically stable and did not require any preconditioning before the application of the lipid solutions. The adhesion of the polyimide to the sacrificial silica layer was excellent.

BLM formation

The polyimide septum was completely dried at about 80°C in an oven before BLM formation. Then 7 μl of lipid solution was gently dropped over the aperture using a micropipette holder. Within the next twenty seconds, after the lipid solution was applied to the aperture, both chambers of the Teflon fluoropolymer cuvette were filled up very slowly with appropriate salt solutions. Over about the next thirty seconds, thinning of the BLM could be observed with an increasing capacitance. Collective capacitance of the polyimide septum and BLM stabilized at 220 pF. After a month of experiments, the aperture was plugged with a small amount of polyimide and cured. Then, the intrinsic capacitance of the septum was measured to be 200 pF. This gives a specific capacitance of 1.59 $\mu F/cm^2$ for the BLM as compared to Tien, in Bilayer Lipid Membranes Theory and Practice (Marcel Dekker, New York, 1974), who reported specific capacitance values of 0.3-0.8 $\mu F/cm^2$. The discrepancy might be due to measurement inaccuracies. The specific resistance was

measured to be 1.98 x 10^{15} Ω/cm^2. This figure slightly increased with time which might have been due to improved packing of head groups of lipid molecules. A stability experiment yielded a lifetime of 50 hours under an applied 125 Hz, 20 mV_{p-p} triangular signal which is believed to be a record for suspended BLMs.

Alamethicin activity

Alamethicin is a membrane spanning ion channel. If the membrane is not a bilayer, the alamethicin activity cannot be observed (Alvarez et al., supra, 1981). In this experiment, both trans and cis sides of the BLM contained 0.5 M NaCl (pH = 7.4) solutions. Under constant stirring rates, 2 μl of alamethicin stock solution was applied to the cis side of BLM. Within about the next three minutes, channel activity was observed. Part of the data recorded at a +150 mV holding potential (cis side positive) is given in Fig. 6. Three main activity levels with conductivities of 61 pU, 85 pU, and 115 pU, respectively, were observed. Multiple conduction states (nine of them) for alamethicin channels were previously observed by Latorre et al., Acta Physiol. Scand. Suppl. 481, 37-41 (1980). It is believed that flickering behavior of close level indicates that many channels are trying to activate. When a channel is open, flickering is much less severe.

Vesicle-BLM fusion and AChR activity

Before the experiments, AChR stock solution was thawed to room temperature and was very gently sonicated. Both sides of the BLM were composed of 20 mM potassium chloride and 10 mM hepes (pH - 7.4). Then, 2 μl of stock solution was added to the cis side of the BLM under constant stirring conditions and +150 mV applied potential. Within the next ten minutes, no activity was observed. Next, 5 μM of calcium

chloride was added to the cis side to promote fusion by attracting the vesicles to the vicinity of BLM, a state called perfusion by Cohen et al. in J. Cell. Biol. 98, 1054-1062 (1984). Still, no activity was observed within the next ten minutes. Finally, an osmotic gradient of 200 mM was established between the cis and trans sides which resulted in vesicle-BLM fusion within a few minutes. The osmotic gradient was immediately terminated and stirring was stopped during recording. Fig. 7 shows the AChR activity. Two main activities with conductances of 61 pÜ, and 140 pÜ were observed which are believed to be the sub- and main conductance levels of a single AChR molecule. Similar activity for AChRs in the synaptic membrane of skeletal muscle was observed by Hamill et al. in Nature 294, 462-464 (1981) with conductance levels of 25 and 35 pÜs.

The foregoing describe certain preferred embodiments of the present invention for illustrative purposes only and should not be construed in a limiting sense. The scope of protection sought is set forth in the claims which follow.

Claims

1. A biosensor substrate, adapted to mount a bilayer lipid membrane which contains at least one receptor, which comprises:

(a) a body having side walls and a solid floor which define a chamber to hold a first solution;

(b) at least one open aperture in the solid floor which is of suitable dimensions to allow for the structural integrity of the bilayer lipid membrane comprising the receptor when the membrane is placed across the open aperture in contact with the first solution; and

(c) a chamber below the solid floor adapted to hold a second solution in contact with the membrane.

2. A substrate as claimed in Claim 1 wherein the floor is formed of silicon.

3. A substrate as claimed in Claim 1 wherein the receptor is an ion channel receptor.

4. A substrate as claimed in Claim 2 wherein the receptor is an ion channel receptor.

5. A substrate as claimed in Claim 1 wherein the aperture is round.

6. A substrate as claimed in Claim 2 wherein the aperture is round.

7. A substrate as claimed in Claim 3 wherein the aperture is round.

8. A substrate as claimed in Claim 4 wherein the aperture is round.

9. A substrate as claimed in Claim 1 wherein the aperture is round and has a diameter of from about 1 μm to about 50 μm.

10. A substrate as claimed in Claim 2 wherein the aperture is round and has a diameter of from about 1 μm to about 50 μm.

11. A substrate as claimed in Claim 3 wherein the aperture is round and has a diameter of from about 1 μm to about 50 μm.

12. A substrate as claimed in Claim 4 wherein the aperture is round and has a diameter of from about 1 μm to about 50 μm.

13. A substrate as claimed in Claim 1 wherein the floor is formed of polyimide.

14. A substrate as claimed in Claim 3 wherein the floor is formed of polyimide and the aperture is round.

15. A substrate as claimed in Claim 3 wherein the floor is formed of polyimide and the aperture is round and has a diameter of from about 1 μm to about 50 μm.

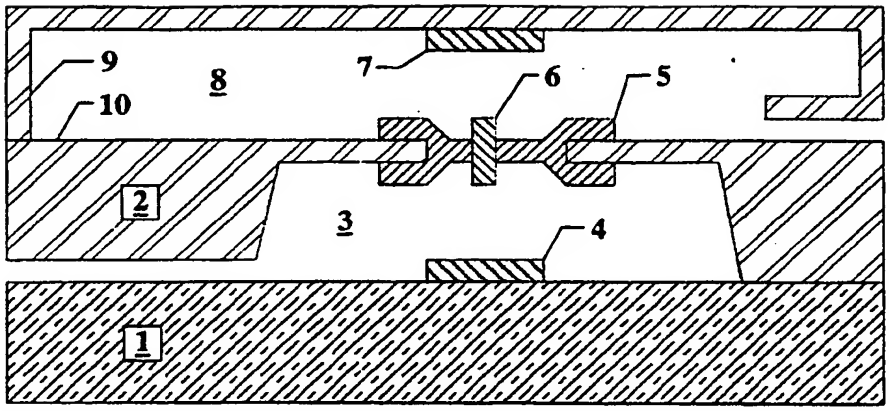

FIG. 1

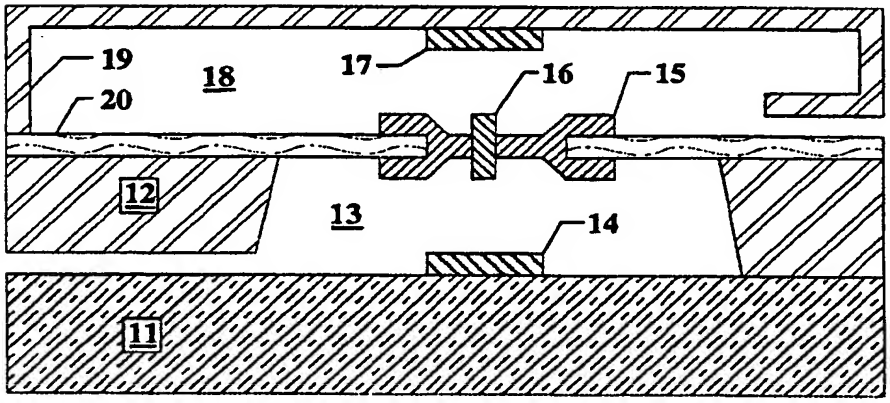

FIG. 2

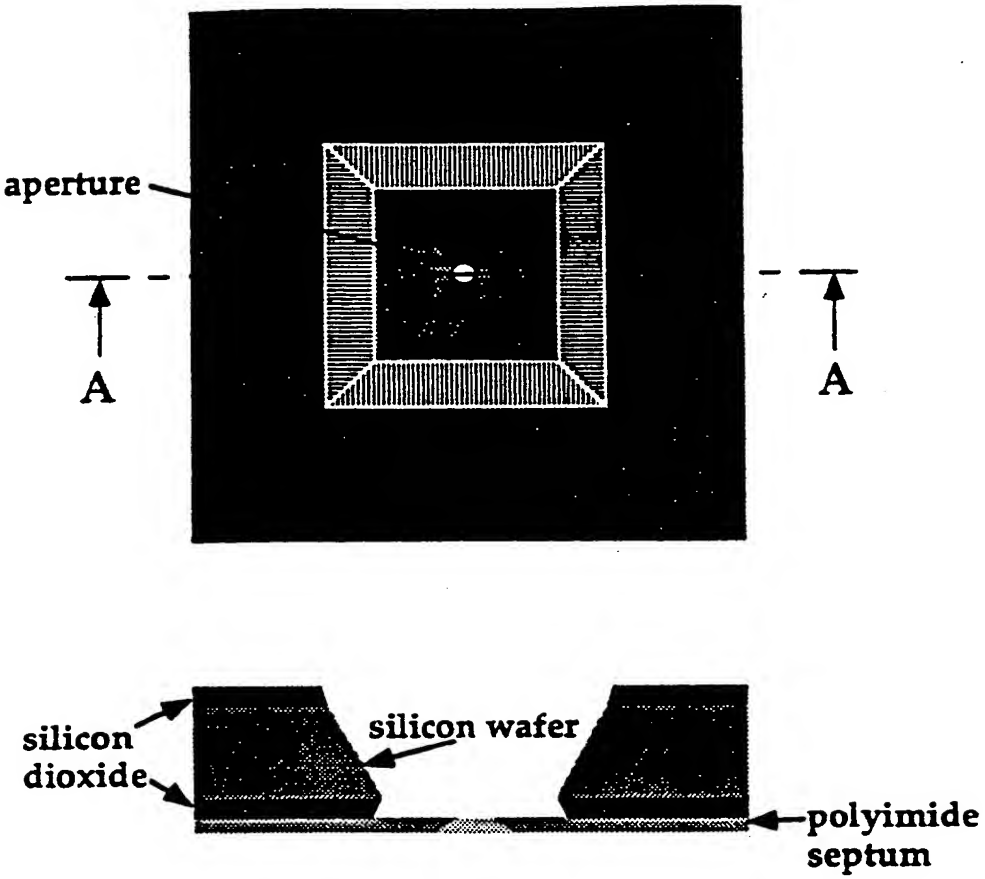
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Fig. 3

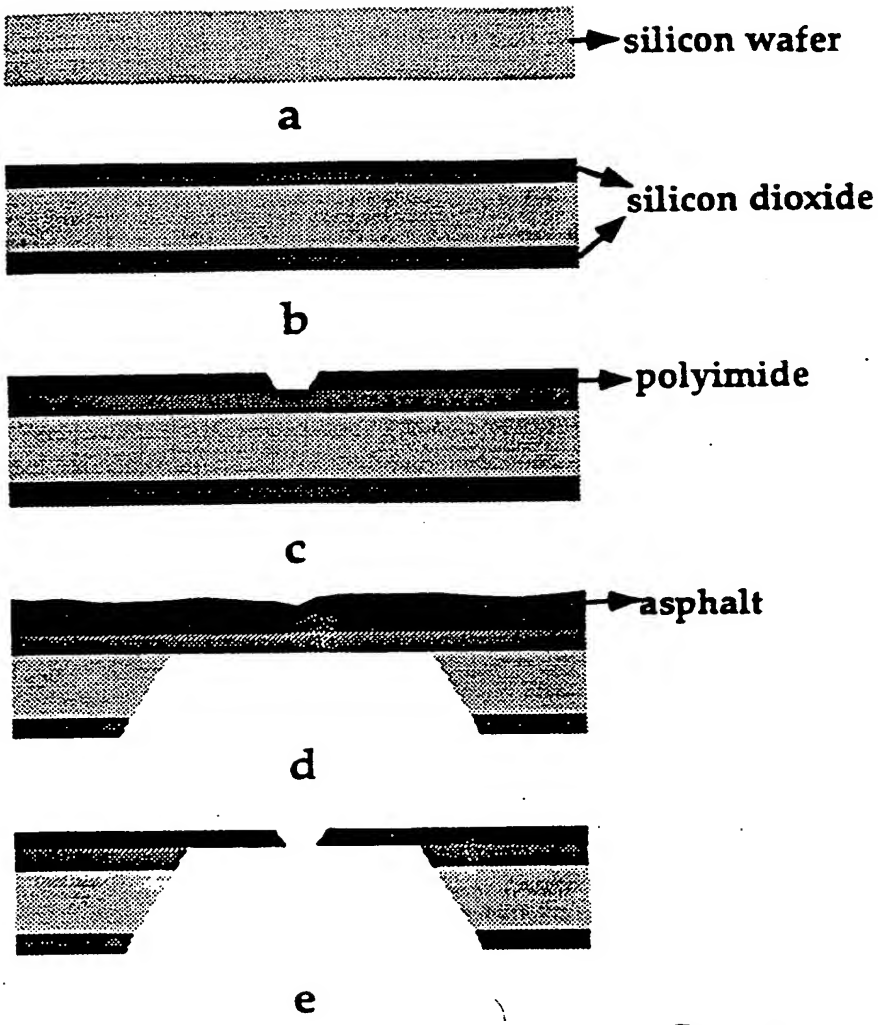


Fig. 4

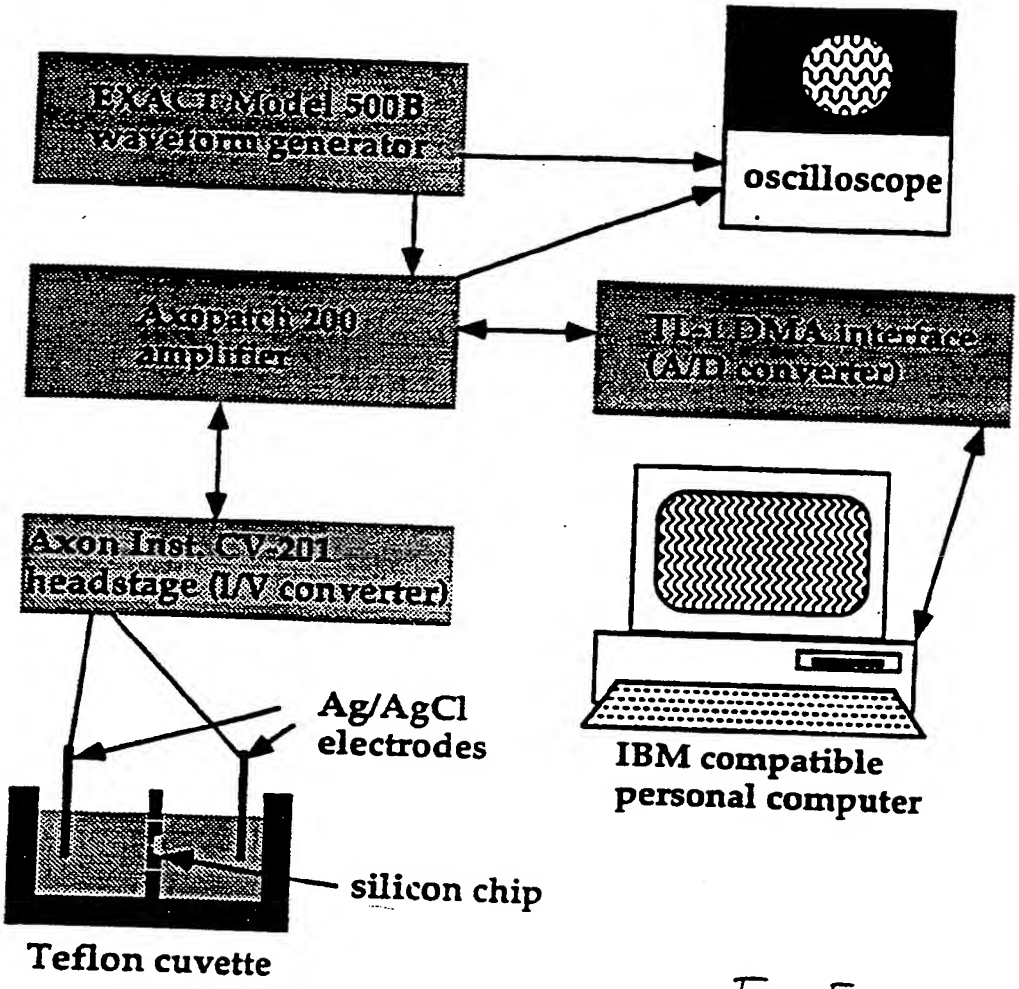

EXACT Model 500B waveform generator
oscilloscope
Axopatch 200 amplifier
TL-1 DMA interface (A/D converter)
Axon Inst. CV-201 headstage (I/V converter)
Ag/AgCl electrodes
IBM compatible personal computer
silicon chip
Teflon cuvette

Fig 5

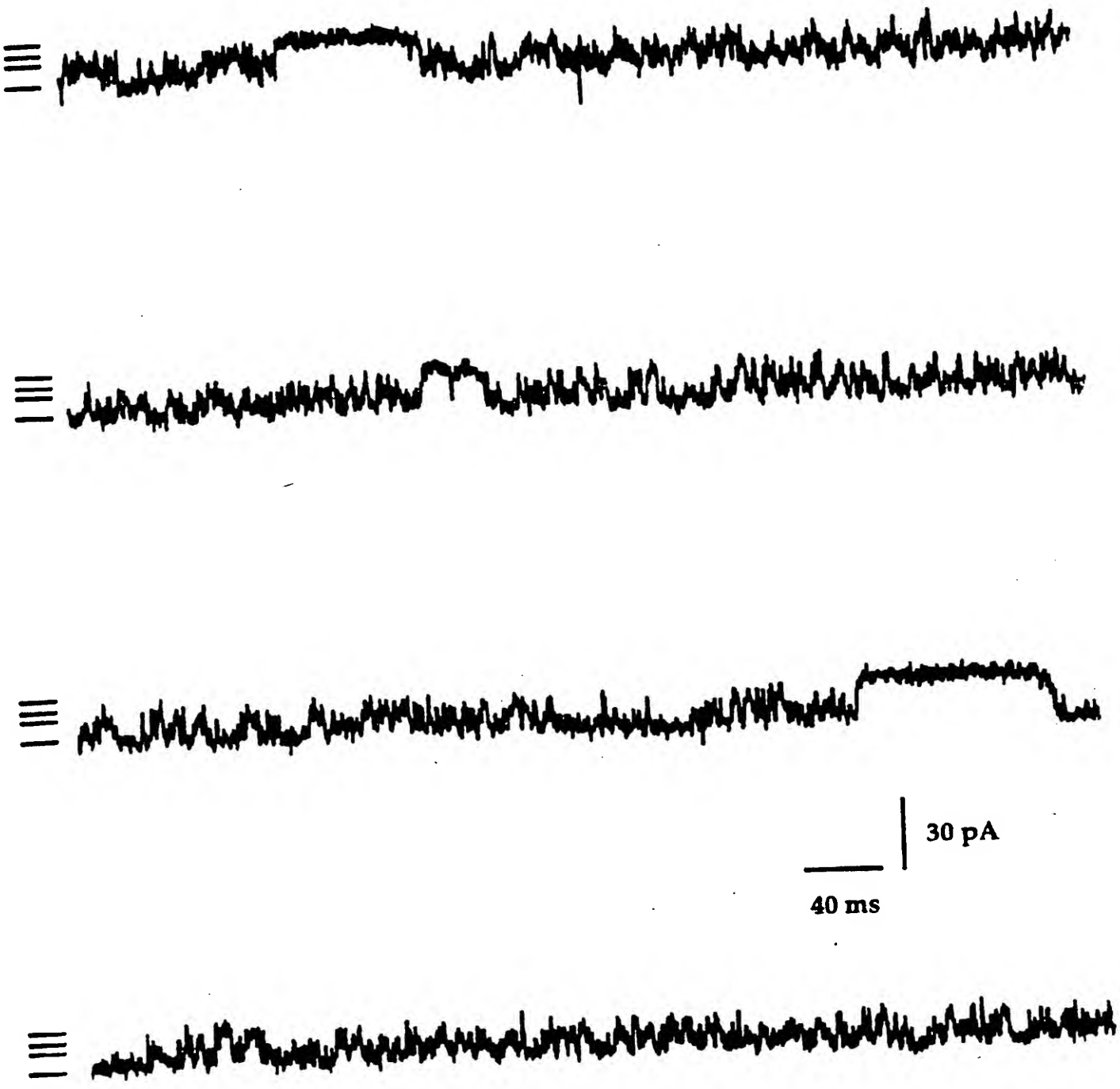
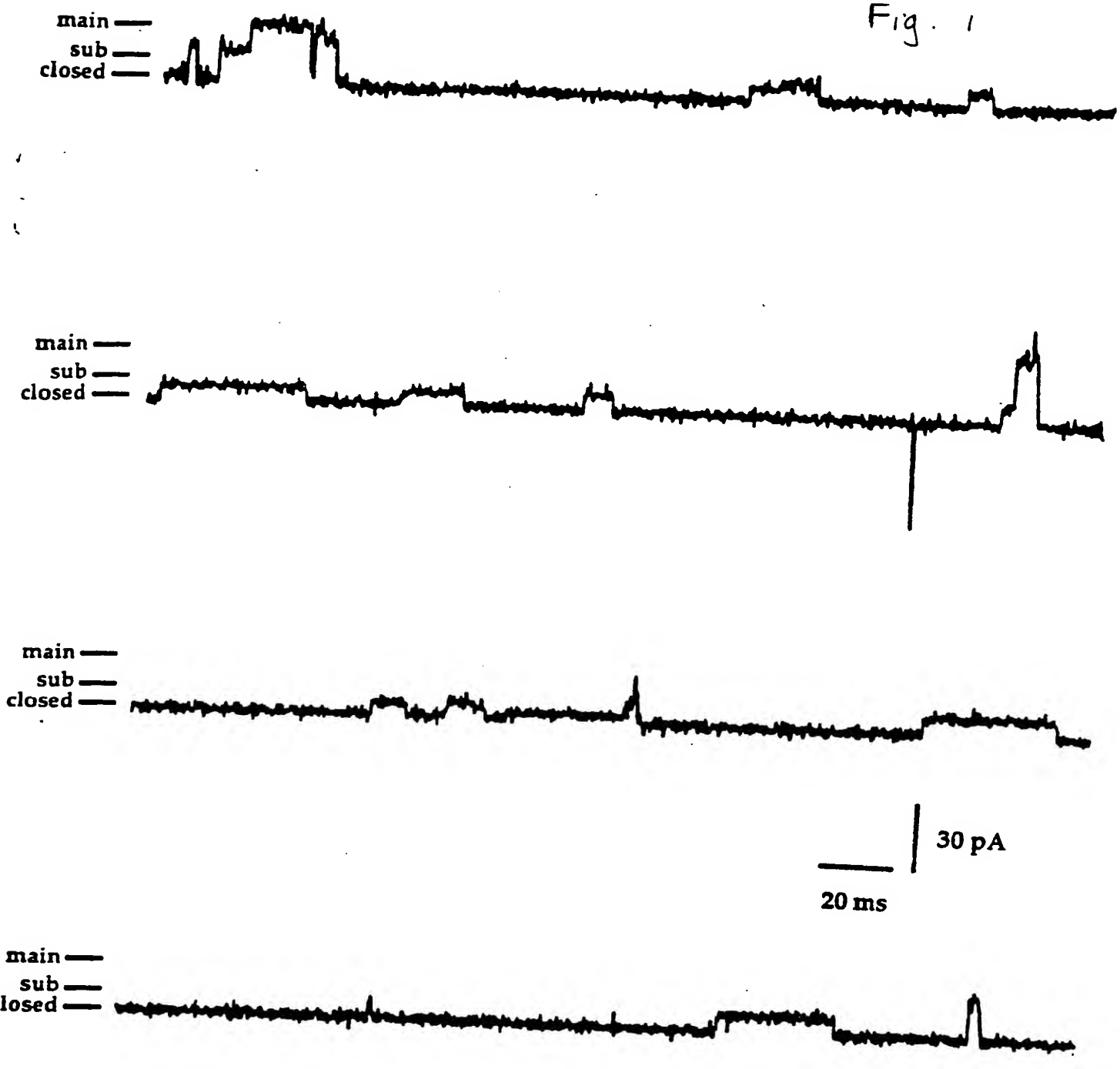


Fig. 1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INTERNATIONAL SEARCH REPORT

In. ..ational application No.
PCT/US94/04883

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) : G01N 27/333
US CL : 204/403, 416, 418
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 204/403, 415, 416, 418,419

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,H, 201 (Yager) 06 January 1987, see column 4, line 60 to column 5, line 23.	9-12
X ---- Y	US,A, 3,856,633 (Fletcher, III) 24 December 1974, see figure 2 and column 4, lines 14-36.	1 and 3 ---------- 1-15
Y	US,A, 4,456,522 (Blackburn) 26 June 1984, see column 2, line 57 to to column 3, line 9.	13-15
X ---- Y	US, A, 4,661,235 (Krull et al) 28 April 1987, see figures 4 and 5 and column 6, line 63 to column 7, line 62.	1,3,5 and 7 ---------- 1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04883

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A, 4,874,499 (Smith et al) 17 October 1989, see column 4, line 42-46 and column 5, lines 43-46.	2,4,6,8,10 and 12
A	US,A, 5,001,048 (Taylor et al) 19 March 1991, see column 6, line 43 to column 7, line 2.	1-15
A	US,A, 5,111,221 (Fare et al) 05 May 1992, see column 4, line 30 to column 6, line 68.	1-15